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Neuraminidase Receptor Binding Variants of Human Influenza A(H3N2) Viruses Resulting from Substitution of Aspartic Acid 151 in the Catalytic Site: a Role in Virus Attachment?▽

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Changes in the receptor binding characteristics of human H3N2 viruses have been evident from changes in the agglutination of different red blood cells (RBCs) and the reduced growth capacity of recently isolated viruses, particularly in embryonated eggs. An additional peculiarity of viruses circulating in 2005 to 2009 has been the poor inhibition of hemagglutination by postinfection ferret antisera for many viruses isolated in MDCK cells, including homologous reference viruses. This was shown not to be due to an antigenic change in hemagglutinin (HA) but was shown to be the result of a mutation in aspartic acid 151 of neuraminidase (NA) to glycine, asparagine, or alanine, which caused an oseltamivir-sensitive agglutination of RBCs. The D151G substitution was shown to cause a change in the specificity of NA such that it acquired the capacity to bind receptors, which were refractory to enzymatic cleavage, without altering its ability to remove receptors for HA. Thus, the inhibition of NA-dependent agglutination by the inclusion of oseltamivir carboxylate in the assay was effective in restoring the anti-HA specificity of the hemagglutination inhibition (HI) assay for monitoring antigenic changes in HA. Since the NA-dependent binding activity did not affect virus neutralization, and virus populations in clinical specimens possessed, at most, low levels of the "151 mutant," the biological significance of this feature of NA in, for example, immune evasion is unclear. It is apparent, however, that an important role of aspartic acid 151 in the activity of NA may be to restrict the specificity of the NA interaction and its receptor-destroying activity to complement that of HA receptor binding.

A characteristic feature of human influenza viruses is their frequent antigenic change to evade host immunity and cause recurrent annual epidemics of disease. As a consequence, available vaccines do not confer long-term immunity, and their composition is regularly reviewed by the WHO Global Influenza Surveillance Network (GISN) and updated to reflect changes in the antigenic characteristics of circulating viruses (2, 43).

The two surface glycoproteins of the virus, hemagglutinin (HA) and neuraminidase (NA), perform clearly defined complementary roles in virus infection. Virus HA is responsible for the attachment of virus to sialic acid-containing glycoconjugates on susceptible cells, and it is antibody to HA, which neutralizes virus infectivity, that is of prime importance in immunity (37). Antibody to NA also contributes to the suppression of disease (3, 16). NA is responsible for destroying receptors for HA by removing the terminal sialic acid moieties from, and thereby inactivating, potentially inhibitory molecules such as mucins in the respiratory tract and from receptors on the surface of virus-infected cells to promote the release of progeny virus, thereby aiding virus transmission (1, 21, 26, 34). Thus, since NA may also cleave receptors from target cells, the maintenance of a balance between the receptor binding and receptor-destroying properties of HA and NA, respectively, is important in optimizing their respective functions in virus replication and maintaining epidemic potential (29, 41).

Virus neutralization is principally the result of the inhibition of the attachment of HA to its receptor (9), and the hemagglutination inhibition (HI) assay is a simple and generally robust surrogate assay for monitoring antigenic relationships among viruses and is the principal basis for changes in vaccine composition recommended by the WHO (2, 43). Many mutations resulting in antibody escape cause amino acid substitutions close to the HA receptor binding site, which may influence receptor binding affinity and/or specificity as well as antigenicity (7, 37, 44). In turn, changes in receptor avidity and binding characteristics of HA, possibly associated with antigenic changes, may influence the effectiveness of the antibody inhibition of the agglutination of red blood cells (RBCs) in the standard HI assay and thereby complicate the interpretation of antigenic relationships (8, 12, 44).

Antigenic drift among H3N2 viruses has been more muted in recent years. Whereas the antigenic drift of viruses between 1992 and 1997 required four changes in the H3N2 vaccine component, there was little progressive antigenic change in the HAs of A/Sydney/5/97(H3N2)-like viruses during the subsequent 5 years prior to the emergence of the A/Fujian/411/2002(H3N2)-like viruses (20) or among the more recently iso-

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lated A/Wisconsin/67/2005(H3N2)-like viruses between 2005 and 2009 (see below). Changes in the receptor binding characteristics of HA have been apparent from changes in the spectrum of RBCs agglutinated by the viruses, e.g., the loss of agglutination of chicken RBCs by H3N2 viruses circulating in the early 1990s (28, 31) and more recently by the poorer growth characteristics following the emergence of A/Fujian/411/2002-like viruses, particularly in embryonated eggs (22), which has "hampered" the selection of suitable vaccine candidates. Amino acid substitutions in residues 190 and 226 in the HA receptor binding site were implicated in the changes in hemagglutination (28, 31), while changes that increased the receptor binding of HA or decreased the enzyme activity of NA were shown to increase the growth of virus in eggs (22). Studies of differences among H3N2 viruses in their relative abilities to bind to and elute from RBCs of different species led Gulati et al. (11) to conclude that the more recently isolated Fujian/411/2002-like viruses bound different forms of sialic acid, which were not cleaved by the virus enzyme. However, studies using glycan arrays failed to identify any differences in receptor binding specificities, or in the amino acid sequences, of HAs that correlated with differences in hemagglutination (18).

Another peculiar feature of many MDCK cell isolates of A/Wisconsin/67/2005-like viruses, isolated between 2005 and 2009, has been the poor inhibition of agglutination of turkey (and guinea pig) RBCs by reference postinfection ferret antisera, with the consequent difficulty in interpreting antigenic relationships from HI data (as reported herein). Here we describe the results of a series of experiments that demonstrate that this phenomenon is not due to changes in antigenicity or simply to changes in HA receptor binding but is the result of the selection in MDCK cells of changes in NA that promote NA-dependent, NA inhibitor-sensitive hemagglutination, which is refractory to inhibition by anti-HA antibody. The replacement of aspartic acid 151 of NA by glycine, which did not affect significantly the activity of the enzyme or its ability to remove receptors for HA, was shown to alter the specificity of NA, resulting in the attachment of virus via its NA to sialic acid receptors refractory to catalytic cleavage.

MATERIALS AND METHODS

Cells. Modified Madin-Darby canine kidney (MDCK-SIAT1) cells were kindly provided by M. Matrosovich, Marburg, Germany. The cells were stably transfected with the human CMP-*N*-acetylneuraminate:β-galactoside α-2,6-sialyltransferase gene for the enhanced expression of Neu5Ac2-6Gal-terminated oligosaccharides (24). Cells were propagated in Dulbecco's modified Eagle's medium (DMEM) (catalog number D6429; Sigma), supplemented with 10% heat-inactivated fetal calf serum (FCS), the antibiotics penicillin (100U/ml) and streptomycin (100 μg/ml), and 1 mg/ml G418 sulfate (Geneticin; Gibco), at 37°C with 5% CO_2. Human embryonic kidney 293T cells and unmodified MDCK cells were cultured under the same conditions but without G418.

Viruses. The influenza viruses used in this study (except recombinants) were from stocks held in the WHO Collaborating Centre for Reference and Research on Influenza (WHO CC), Mill Hill, London, United Kingdom. These viruses were originally isolated from clinical samples by the National Influenza Centres, mostly in cell culture, and were further propagated in either MDCK or MDCK-SIAT1 cells or, for a few viruses only, in 10-day-old embryonated hen eggs. Passage histories, given in Tables 1, 2, and 5, indicate passage in the laboratory supplying the viruses, followed by passages in the WHO CC. For the plaque reduction assays, all virus samples were aliquoted and stored at −80°C before use in successive experiments. The clinical specimens used for pyrosequencing analyses were provided by W. Lim, National Influenza Centre, Hong Kong.

Recombinant viruses, in which the HA and/or NA gene of A/WSN/33 was replaced by those of H3N2 viruses, were generated by reverse genetics, as previously described by Hoffmann et al. (13). Briefly, viral RNA was isolated by using QIAamp viral RNA kits, and one-step reverse transcription (RT)-PCR was performed by using SuperScript III reverse transcriptase and Platinum *Pfx* DNA polymerase. Primers Bm-HA-F1, AarI-HA-F1, Bm-NS-R890, and AarI-NS-R890 were used for amplifying HA genes, and primers Ba-NA-F1 and Ba-NA-R1413 were used for amplifying NA genes. The sequences of the primers are available upon request. The amplification program was one cycle at 50°C for 30 min and one cycle at 94°C for 10 min followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 2.5 min and then 68°C for 10 min. After digestion of the PCR products with BsmBI (Bm), AarI, and BsaI, as required, the fragments were cloned into vector pHW2000. Mutations were introduced into cDNA clones by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Eight plasmids, each containing one of the eight virus genes, were cotransfected into cocultured 293T and MDCK-SIAT1 cells. After 3 to 5 days, viruses in the supernatants of transfected cells were recovered by passage in MDCK-SIAT1 cells or in embryonated eggs.

HI assays. Hemagglutination and HI assays were performed according to standard methods by using suspensions of guinea pig (1.0%), human type O (1.0%), chicken (0.75%), or turkey (0.75%) RBCs. Four HA units were used for HI tests. For HI tests including 20 nM oseltamivir carboxylate, HA titers were determined in the presence of drug. HI titers are reciprocals of the highest dilution of sera that inhibited hemagglutination.

Antisera, including postinfection ferret antisera against various H3N2 reference viruses, hyperimmune rabbit antiserum against the reassortant virus A/equine/Prague/56 (HA) × A/Moscow/10/99 (NA) (H7N2), and hyperimmune sheep antiserum against purified HA from A/Wisconsin/67/2005 (provided by J. Wood, National Institute for Biological Standards and Control, United Kingdom), were treated with receptor-destroying enzyme from *Vibrio cholerae*.

Various bacterial neuraminidases, including those from *Vibrio cholerae*, *Clostridium perfringens*, and *Arthrobacter ureafaciens*, were tested for their ability to inhibit the agglutination of RBCs. Briefly, RBCs were incubated with 0.01 to 100 mU of different neuraminidases in 50 μl at 37°C for 1 h before adding 8 HA units of test viruses at 4°C, and agglutination was recorded after 1 h.

Eight HA units of virus were added to serial 3-fold dilutions of α_2-macroglobulin (up to 2 mg/ml) (from human plasma; Sigma), glycophorin (up to 2 mg/ml) (predominantly glycophorin A, from human blood; Sigma), fetuin (up to 10 mg/ml) (from fetal bovine serum; Sigma), or colominic acid (up to 2.5 mg/ml) (from *Escherichia coli*; Sigma). After 10 min at room temperature, an equal volume (50 μl) of 0.75% turkey or 1% guinea pig RBCs was added, and plates were left at room temperature for 1 h before the HA titer was recorded.

Virus neutralization assay. The microtiter plaque reduction assay, performed as described previously by Matrosovich et al. (25), compared 2-fold dilutions of antiserum starting at 1:40, and the neutralization titer was determined as the reciprocal of the dilution of antiserum corresponding to a 50% reduction in plaques compared to the virus control.

NA assays. Unless otherwise specified, the enzyme activity of virus in cell culture medium was measured by using the fluorescent substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) and a Jasco FP-6300 fluorimeter with excitation and emission wavelengths of 365 nm and 450 nm, respectively. The kinetic parameters of inhibition by oseltamivir carboxylate, zanamivir, or α2,3- and α2,6-sialyl-lactose were measured by adding the inhibitor to a standard reaction mixture of enzyme and MUNANA approximately 50 to 200 s after the initiation of the reaction. NA activity changes in the presence of inhibitor were monitored as the first derivative of the fluorescence change, and the Michaelis-Menten constants (K_m) and the dissociation constants (K_i) for enzyme-inhibitor complexes were determined, as previously described (5). Relative apparent V_{max} values were obtained by using equivalent HA titers of viruses. The Amplex Red neuraminidase (sialidase) assay kit, incorporating fetuin as a substrate, was obtained from Molecular Probes, Inc., and used according to the manufacturer's instructions.

Nucleotide sequence analyses. Nucleotide sequences of HA and NA genes in viruses, cDNA clones, and recombinant viruses were determined by using ABI Prism BigDye terminator cycle sequencing kits and a MegaBACE 1000 DNA sequencer. Sequence data were edited and analyzed by using the Wisconsin Sequence Analysis package, version 10 (GCG).

For pyrosequencing, to assess the genetic composition of the codon for amino acid residue 151 of N2 NA, primers N2F382/404 (5′-AAGTGTTATCAATTTG CCCTYGG-3′) and BiotN2R883/905 (5′-Biotin-ATGGGCCTATTGGAKCCT TTCCA-3′) were used to generate PCR products from either clinical specimens

TABLE 1. Differences in inhibition by postinfection ferret antisera of the agglutination of turkey RBCs by MDCK cell- and egg-grown viruses[e]

Virus	Collection (mo/day/yr or mo and yr)	Passage history[d]	Hemagglutination inhibition titer of postinfection ferret sera[a]							
			A/Wisconsin/67/05	A/Nepal/921/06	A/Trieste/25c/07	A/Trieste/25e/07	A/Brisbane/10/07	A/Uruguay/716/07	A/Finland/9/08	A/Johannesburg/15/08
Reference viruses										
A/Wisconsin/67/2005[c]	8/31/2005	SpfCk3, E3 E4	**5,120**	5,120	2,560	2,560	2,560	5,120	2,560	2,560
A/Nepal/921/2006[c]	7/11/2006	E2 E2	1,280	**5,120**	2,560	2,560	1,280	2,560	2,560	5,120
A/Trieste/25c/2007[c]	January 2007	MDCKx MDCK4	<40	80	**80**	160	80	80	80	80
A/Trieste/25e/2007[b,c]	January 2007	MDCKx MDCK2,E2	1,280	2,560	1,280	**1,280**	640	2,560	1,280	640
A/Brisbane/10/2007[c]	2/6/2007	E2 E1	1,280	5,120	2,560	2,560	**1,280**	2,560	2,560	1,280
A/Uruguay/716/2007	6/21/2007	SpfCk1, E3 E2	2,560	5,120	2,560	2,560	1,280	**5,120**	2,560	1,280
A/Finland/9/2008[c]	1/7/2008	MDCK2 SIAT3	<40	80	160	160	160	80	**320**	160
A/Johannesburg/15/2008[c]	6/26/2008	MDCKx SIAT2	<40	80	160	160	80	80	160	**160**
Test viruses										
A/Toyama/119/2007	Unknown	Cx SIAT1	160	80	80	80	160	160	ND	ND
A/Paris/0358/2007	November 2007	MDCKx SIAT1	160	80	80	160	320	160	ND	ND
A/Nevada/05/2007	10/5/2007	MDCK2 SIAT1	80	80	80	160	320	160	ND	ND
A/Geneva/8428/2009[c]	1/22/2008	MDCK3 SIAT1	40	80	40	80	160	80	ND	ND
A/Finland/149/2008	1/28/2008	MDCK2 SIAT1	<40	80	160	160	80	40	160	80
A/Ulaanbaatar/1600/2008	2/13/2008	C2/C2 SIAT1	40	80	160	160	160	80	80	160
A/Lyon/762/2008	2/26/2008	MDCK2 SIAT1	40	80	160	160	80	40	80	80
A/Hong Kong/796/2008[c]	3/15/2008	MDCK2 SIAT1	320	160	160	320	640	160	320	ND
A/Denmark/190/2008[c]	4/17/2008	MDCK2 SIAT1	<40	80	160	160	80	40	160	80
A/Hong Kong/1050/2008[c]	4/23/2008	MDCK2 SIAT1	<40	160	160	160	80	80	80	80
A/Lyon/CHU/19.113/2008[c]	5/5/2008	MDCK2 SIAT1	<40	<40	40	80	160	80	40	ND
A/Mauritius/402/2008[c]	7/9/2008	SIAT2 SIAT1	<40	80	80	80	40	40	40	40
A/Ghana/11/2008[c]	7/15/2008	MDCKx SIAT1	40	80	80	160	160	160	160	160
A/Hessen/6c/2007[c]	2/9/2007	MDCKx SIAT1	40	40	40	40	80	80	40	ND
A/Berlin/20c/2007[c]	2/9/2007	MDCKx SIAT1	<40	40	40	40	160	80	80	ND
A/Hessen/6e/2007[b,c]	2/9/2007	MDCKx E3	640	1,280	1,280	1,280	1,280	1,280	1,280	ND
A/Berlin/20e/2007[b,c]	2/9/2007	MDCKx E3	1,280	1,280	1,280	1,280	1,280	1,280	1,280	ND
A/Wisconsin/3/2007	1/21/2007	Ex E6	5,120	5,120	5,120	2,560	2,560	5,120	2,560	2,560
A/Singapore/21/2008	5/2/2008	E3 E1	1,280	2,560	1,280	1,280	640	2,560	1,280	640
A/Wellington/2/2008	5/28/2008	E3 E1	2,560	1,280	2,560	1,280	2,560	2,560	1,280	2,560
A/Brisbane/24/2008	6/23/2008	E5 E1	2,560	2,560	2,560	2,560	1,280	5,120	2,560	2,560

[a] ND, not done.
[b] Viruses isolated in MDCK cells and subsequently passaged in eggs.
[c] Viruses for which neutralization data are included in Table 2.
[d] Abbreviations: SpfCk, specific-pathogen-free chick kidney cells; E, embryonated egg; C, cell culture (unspecified); SIAT, MDCK-SIAT1 cells.
[e] Homologous titers are shown in boldface type.

TABLE 2. Virus neutralization by postinfection ferret antisera[a]

Virus	Passage history[b]	Neutralization titer of postinfection ferret sera[c]							
		A/Fujian/411/02	A/Wellington/1/04	A/Wisconsin/67/05	A/Nepal/921/06	A/Brisbane/10/07	A/Trieste/25c/07	A/Trieste/25e/07	A/Finland/9/08
Reference viruses									
A/Fujian/411/2002	MDCKx MDCK5	**640**	80	160	160	160	80	ND	160
A/Wellington/1/2004	Ex Ex	160	**2,560**	320	320	320	640	ND	320
A/Wisconsin/67/2005	SpfCk3, E3 E4	160	640	**2,560**	2,560	1,280	2,560	2,560	ND
A/Nepal/921/2006	E2 E2	160	320	1,280	**5,120**	2,560	1,280	1,280	ND
A/Brisbane/10/2007	E2 E1	80	640	1,280	2,560	**2,560**	1,280	1,280	ND
A/Trieste/25c/2007	MDCKx MDCK4	80	320	1,280	2,560	2,560	**2,560**	1,280	ND
A/Trieste/25e/2007	MDCKx MDCK2, E2	160	320	2,560	1,280	1,280	2,560	**1,280**	ND
A/Finland/9/2008	MDCK2 SIAT3	80	160	640	1,280	1,280	1,280	1,280	**2,560**
Test viruses									
A/Berlin/20c/2007	MDCKx SIAT3	80	160	640	1,280	1,280	1,280	ND	640
A/Berlin/20e/2007	MDCKx E3	160	640	2,560	2,560	2,560	5,120	ND	ND
A/Hessen/6c/2007	MDCKx SIAT3	160	640	1,280	5,120	2,560	ND	2,560	ND
A/Hessen/6e/2007	MDCKx E3	80	320	1,280	2,560	1,280	ND	1,280	ND
A/Geneva/8428/2008	MDCK3 SIAT1	80	80	320	1,280	640	640	ND	ND
A/Hong Kong/796/2008	MDCK2 SIAT1	160	320	1,280	2,560	2,560	2,560	ND	2,560
A/Hong Kong/1050/2008	MDCK2 SIAT1	160	320	1,280	2,560	1,280	2,560	ND	1,280
A/Lyon/CHU/19.113/2008	MDCK2 SIAT1	160	320	1,280	2,560	2,560	2,560	ND	2,560
A/Johannesburg/15/2008	MDCKx SIAT2	160	160	640	2,560	1,280	2,560	ND	1,280
A/Mauritius/402/2008	SIAT2 SIAT1	80	160	1,280	1,280	1,280	2,560	ND	1,280

[a] Homologous titers are shown in boldface type.
[b] Abbreviations: SpfCk, specific-pathogen-free chick kidney cells; E, embryonated egg; C, cell culture (unspecified); SIAT, MDCK-SIAT1 cells.
[c] ND, not done.

or virus isolates. Subsequent sequencing and analysis were performed with primer N2F424/441 (5′-AACGTRCATTCAAATRAC-3′) with a Qiagen PyroMark Q96MD instrument.

RESULTS

Hemagglutination inhibition. Following the emergence of A/Wisconsin/67/2005-like viruses during 2005, there was a marked increase in the proportion of viruses grown in MDCK cells that, in HI tests using turkey RBCs, showed a poor inhibition of agglutination by postinfection ferret antisera compared with egg-grown reference viruses (Table 1). Furthermore, MDCK cell-grown reference viruses, such as A/Trieste/25c/2007, A/Finland/9/2008, and A/Johannnesburg/15/2008, also gave low homologous HI titers. However, following subsequent passage in eggs, A/Trieste/25e/2007 and other viruses, such as A/Hessen/6e/2007 and A/Berlin/20e/2007, showed a pattern of reactivity in HI tests similar to those of contemporary egg-grown reference viruses. In addition, antisera raised against egg- or MDCK cell-passaged viruses, including those against A/Trieste/25c/2007 and A/Trieste/25e/2007, gave similar patterns of hemagglutination inhibition, indicating that the ferret did not recognize any antigenic differences between the various 2005-2008 reference viruses whether grown in eggs or grown in MDCK cells. Furthermore, for many viruses isolated in MDCK cells during 2006 and 2007, hemagglutination was also observed to be poorly inhibited by sheep polyclonal anti-HA serum raised against the closely related A/Wisconsin/67/2005 virus (data not shown).

Virus neutralization. Postinfection ferret antisera that distinguished egg- and MDCK cell-grown viruses in HI (Table 1) failed to distinguish such viruses in virus neutralization assays (Table 2). In particular, viruses such as A/Trieste/25c/2007, A/Berlin/20c/2007, and A/Hessen/6c/2007, which were isolated in MDCK cells, gave patterns of neutralization titers similar to those of the viruses subsequently passaged in eggs, in contrast to the results of HI tests (compare data in Tables 1 and 2). Furthermore, these data showed that viruses isolated from 2005 to 2008, including A/Wisconsin/67/2005 and A/Brisbane/10/2007, were antigenically similar but distinguishable from the earlier prototype vaccine viruses A/Wellington/1/2004 and A/Fujian/411/2002 and that egg-selected changes (see below) did not alter antigenicity. It is apparent from these data that the differences in HI reflected differences in virus binding to the RBCs rather than differences in antigenicity.

Agglutination of RBCs. For viruses isolated in cell culture, variation in the agglutination of RBCs was noted following the emergence of the A/Fujian/411/2002-like viruses (Table 3). MDCK cell isolates, such as A/Fujian/411/2002 and A/Finland/486/2004, like the previous A/Sydney/5/97-like viruses, agglutinated turkey RBCs but failed to agglutinate chicken RBCs. However, many A/Wisconsin/67/2005-like viruses isolated after 2005, such as A/Hong Kong/4443/2005 and A/Trieste/25c/2007, were observed to agglutinate chicken RBCs, while others, particularly among more recent A/Brisbane/10/2007-like (e.g., A/England/233/2009 and A/Cameroon/350/2009) and A/Perth/16/2009-like (e.g., A/Ghana/Fs-09-1238/2009 and A/England/232/2009) viruses (43), failed to agglutinate RBCs from both avian species and human type O RBCs but retained the capacity to agglutinate guinea pig RBCs (Table 3).

In HI tests using guinea pig RBCs, many of the latter viruses, which agglutinated guinea pig but not turkey RBCs, gave patterns of HI titers comparable to those obtained with the egg-grown reference viruses, indicating their antigenic similarity to the vac-

TABLE 3. Variation in agglutination of RBCs from different species by virus isolates and recombinant viruses and susceptibility to oseltamivir

Virus[b]	Residue 151 in NA[c]	Hemagglutination titer of red blood cells[a]							
		Chicken		Turkey		Guinea pig		Human	
		− oseltamivir	+ oseltamivir	− oseltamivir	+ oseltamivir	− oseltamivir	+ oseltamivir	− oseltamivir	+ oseltamivir
Virus isolates									
A/Fujian/411/2002	D	<2	<2	64	32	8	4	32	32
A/Finland/486/2004	D	<2	<2	8	8	32	16	32	32
A/HK/4443/2005	G/D	16	<2	16	<2	16	8	32	2
A/Trieste/25c/2007	G/D	64	<2	64	<2	64	8	64	<2
A/Trieste/25e/2007	D	64	16	64	16	32	8	64	64
A/Hessen/6c/2007	G/D	16	<2	16	<2	64	32	32	8
A/Hessen/6e/2007	D	32	16	64	16	16	8	32	32
A/Parma/1c/2007	D/G	4	<2	32	8	16	8	32	8
A/Parma/1e/2007	D	8	8	16	16	16	4	16	16
A/Paris/1751/2009	N/D	64	<2	128	<2	128	8	128	<2
A/Madagascar/424/2009	N/D	64	<2	128	<2	128	8	128	<2
A/Moldova/169/2009	N/D	64	<2	128	<2	64	8	128	<2
A/Madagascar/878/2009	A/D	16	<2	32	<2	32	8	32	<2
A/Singapore/33/2009[d]	A/D	32	<2	64	<2	64	8	128	<2
A/England/233/2009	D	<2	<2	<2	<2	8	8	<2	<2
A/Cameroon/350/2009	D	<2	<2	<2	<2	4	4	<2	<2
A/Ghana/Fs-09−1238/2009[d]	D	<2	<2	<2	<2	16	8	<2	<2
A/England/232/2009[d]	D	<2	<2	<2	<2	16	8	<2	<2
Recombinant viruses									
RG-Trieste/25(HAc)		ND	ND	<2	<2	256	256	ND	ND
RG-Trieste/25(HAe)		ND	ND	16	ND	16	ND	ND	ND
RG-Parma/1(HAc)		ND	ND	<2	<2	256	256	ND	ND
RG-Parma/1(HAe)		ND	ND	64	128	128	64	ND	ND
RG-Parma/1(HAc/NAc)	D	<2	<2	<2	<2	256	256	64	64
RG-Trieste/25(HAc/NA151D)	D	<2	<2	<2	<2	128	128	<2	<2
RG-Trieste/25(HAc/NA151G)	G	64	<2	64	<2	128	32	32	<2
RG-HK/4443(HA/NA151D)	D	<2	<2	<2	<2	256	256	<2	<2
RG-HK/4443(HA/NA151G)	G	128	<2	128	<2	256	64	128	<2
RG-Finland/486(HAc/NAc)	D	<2	<2	64	128	256	256	256	256

[a] ND, not done.
[b] All viruses were passaged in MDCK or MDCK-SIAT1 cells unless otherwise indicated.
[c] Predominant residues.
[d] A/Perth/16/2009-like.

cine strain A/Brisbane/10/2007 (or A/Uruguay/716/2007) or the more recent A/Perth/16/2009 strain (data not shown).

As expected, egg isolates agglutinated chicken as well as turkey RBCs. However, whereas HA titers with the two species of cells were similar for the A/Sydney/7/97 and A/Moscow/10/99 reference (vaccine) viruses, titers with chicken cells were lower and virus eluted more rapidly, within 30 to 60 min at room temperature, for "post-Fujian" reference viruses, suggesting a lower avidity of binding by the latter viruses.

Sequence comparisons of HA and NA. Two principal clades of viruses, distinguished by their HA and NA sequences, emerged during 2005 (Fig. 1). One clade, which subsequently became dominant in 2006 and is represented antigenically by A/Wisconsin/67/2005 and distinguished from the earlier A/California/7/2004-like (and A/Wellington/1/2004-like) virus by the characteristic amino acid changes S193F and D225N in HA and D93N in NA, remained fairly homogeneous in HA and NA sequences during 2005 and 2006. A number of viruses, particularly from Hong Kong, were observed to have an additional amino acid substitution, D151G, in NA. The other clade, represented by, e.g., A/Berlin/2/2006, was characterized by the common changes V112I and K173E in HA and H150R, V194I, Y310H, L370S, S372L, and N387K in NA and exhibited greater HA and NA sequence diversity. Although viruses of this clade were displaced by A/Wisconsin/67/2005-like viruses during 2006, reassortant viruses, such as A/Nordrhein-Westfalen/7/2007, emerged with a Wisconsin/67/05 clade HA and an NA similar to that of the Berlin/2/06 clade. A similar NA was retained in the subsequently dominant A/Brisbane/10/2007-like and A/Perth/16/2009-like viruses (43).

Egg-selected changes in HA and NA sequences. Seven MDCK cell isolates, representative of different phylogenetic clades, were passed two to three times in eggs. In six of these isolates, the HA acquired the substitution L194P, which was observed to be a common feature of egg isolates; three isolates possessed only this change in HA, including A/Hessen/6e/2007 and A/Berlin/20e/2007, which therefore appears to correlate with increased HI titers (Table 1). The other virus, A/Parma/1e/2007 (with a Nepal/921/06 clade HA), acquired two changes, H156R and K158N, in HA and no change in NA. No common changes were observed in the NA sequences, although for viruses possessing predominantly G151, such as A/Trieste/25c/2007 and A/Hessen/6c/2007, egg passage resulted in the "reversion" G151D.

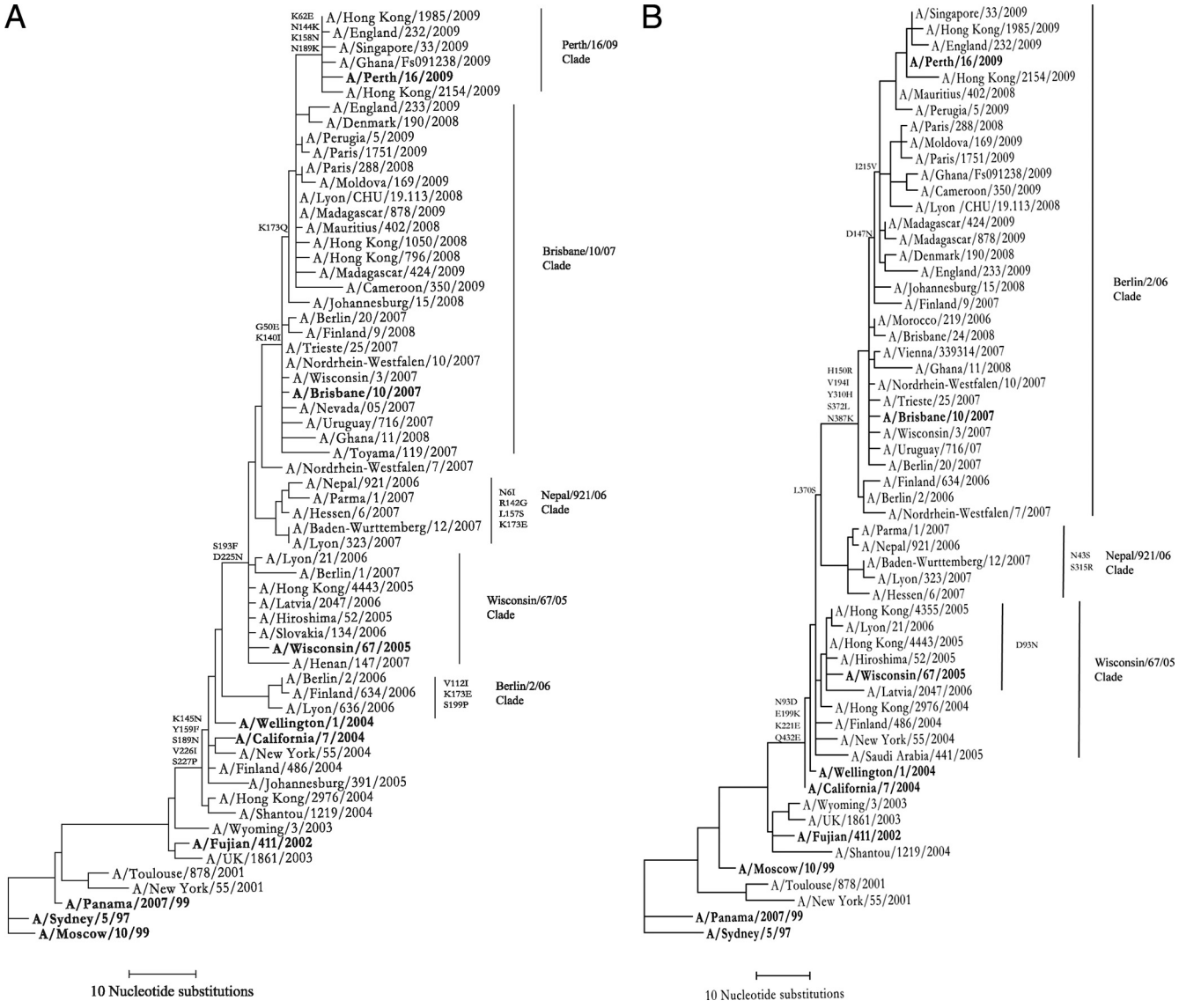

FIG. 1. Phylogenetic comparisons of the HA (A) and NA (B) genes of recently isolated H3N2 viruses. The nucleotide sequences of the HA (nucleotides 177 to 989) and NA (nucleotides 214 to 1369) genes were compared by using maximum parsimony (PAUP, version 4.0; Sinauer Associates, Sunderland, MA). The amino acid substitutions accumulated since the emergence of Fujian/411/2004-like viruses are indicated on the trunk of the tree, and those that distinguish different clades are indicated at the side. Prototype vaccine viruses are indicated in boldface type.

Variation in residue 151 of NA. It was observed that the NA sequences of several viruses possessed G, N, or A amino acid substitutions for D151, while a close examination of the nucleotide sequence traces indicated that many viruses possessed polymorphisms in the codon for residue 151, yielding mixed populations with D and G, D and N, or D and A. Furthermore, variation in this residue appeared to correlate with differences in HI reactivity, with the presence of G, N, or A at position 151 being associated with patterns of low HI titers, such as those shown for viruses in Table 1, and the ability to agglutinate turkey (and chicken) RBCs (Table 3). The proportions of D151 and mutant NAs varied among viruses, and there was no apparent distinction between viruses selected in MDCK-SIAT1 cells, in which viruses grew to higher titers, or the parent MDCK cells from which they were derived.

To investigate the natural significance of these differences in residue 151 of NA and their potential consequences, heterogeneity at this position for viruses in clinical samples, as well as virus isolates, was determined by pyrosequencing. Studies of several clinical specimens from Hong Kong collected between 2000 and 2009 detected only low-level polymorphisms in the codon for residue 151; codons for G or N were detected at levels up to 13% relative to the codon for D. Whereas viruses isolated from the earlier samples (2000 to 2005) after 1 or 2 passages in MDCK-SIAT1 cells yielded viruses with similar low levels, or the absence, of codon polymorphism, many isolates from the more recent 2007-2009 samples showed increases in the proportion of mutations. Analyses of 2008-2009 H3N2 isolates from various geographical locations indicated the presence in virus populations of up to 45% N, 33% G, or

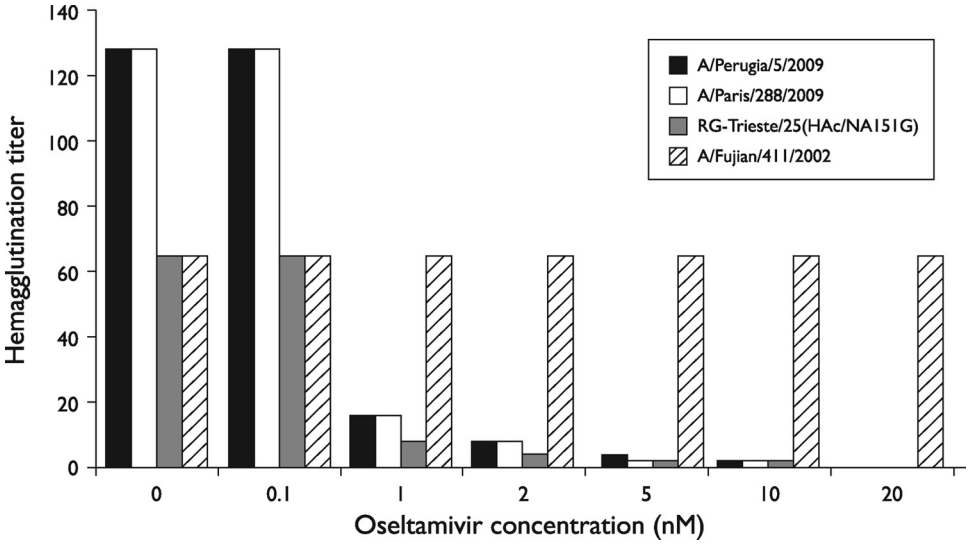

FIG. 2. Concentration dependence of the inhibition of agglutination of turkey RBCs by oseltamivir carboxylate. Three viruses were chosen as representative of those possessing NA-mediated agglutinating activity. A/Fujian/411/2002 was included as a control, representative of pre-Wisconsin/67/2005 viruses.

65% A at position 151 of NA, including amino acid mixtures containing, e.g., both A (65%) and G (27%) in addition to D.

Effects of inhibitors of NA. An initial clue as to the basis of the unusual behavior of MDCK cell-grown viruses in HI titrations was provided by the observation that oseltamivir carboxylate, rather than increasing HA titers, e.g., by inhibiting elution from the RBCs, actually inhibited hemagglutination. This was most marked for turkey (and chicken) RBCs but was also apparent in reductions in HA titers with guinea pig RBCs (Table 3). The concentration dependence of the inhibition of hemagglutination, with a 50% inhibitory concentration (IC_{50}) of approximately 0.2 to 0.5 nM, as illustrated in Fig. 2, was similar to that of the inhibition of the NA enzyme activity of contemporary H3N2 viruses (IC_{50} = 0.1 to 1.0 nM), suggesting that binding via the NA active site was participating in agglutination. Similar results were obtained with zanamivir, in which case the approximately 2- to 3-fold-higher IC_{50} for the inhibition of agglutination was equivalent to that of enzyme inhibition.

As anticipated, anti-NA antibody also inhibited the hemagglutination of turkey RBCs by viruses inhibited by oseltamivir. Table 4 shows a comparison of inhibition by anti-NA antibodies in hyperimmune rabbit anti-H7N2 serum and by anti-HA antibodies in hyperimmune sheep anti-HA (H3) serum. The

TABLE 4. Inhibition of hemagglutination by anti-HA and anti-NA sera

Virus	Residue 151 in NA	Hemagglutination inhibition titer[a]		
		Sheep anti-H3 with guinea pig RBCs		Rabbit anti-H7N2 with turkey RBCs
		− oseltamivir	+ oseltamivir	− oseltamivir
Virus isolates				
A/Baden-Wurttemberg/12c/2007	D	640	640	ND
A/Baden-Wurttemberg/12e/2007[b]	D	320	160	<40
A/Nordrhein-Westfalen/7c/2007	D/G[c]	<40	1,280	320
A/Nordrhein-Westfalen/7e/2007[b]	D	640	640	<40
A/Nordrhein-Westfalen/10c/2007	D/G[c]	<40	2,560	160
A/Nordrhein-Westfalen/10e/2007[b]	D	640	640	<40
A/Parma/1c/2007	D/G[c]	<40	1,280	1,280
A/Parma/1e/2007[b]	D	640	640	<40
Recombinant viruses				
RG-Parma/1(HAc/NAc)	D	2,560	2,560	ND
RG-HK/4443(HA/NA151D)	D	2,560	2,560	ND
RG-HK/4443(HA/NA151G)	G	40	1,280	320
RG-Trieste/25(HAc/NA151D)	D	1,280	2,560	ND
RG-Trieste/25(HAc/NA151G)	G	<40	1,280	80

[a] ND, not done, as virus did not agglutinate turkey RBCs.
[b] Viruses isolated in MDCK cells and subsequently passaged in eggs.
[c] Mixed population.

TABLE 5. Effect of oseltamivir on HI titration using guinea pig RBCs

Virus	Collection date (mo/day/yr or mo and yr)	Passage history[c]	20 nM oseltamivir	Hemagglutination inhibition titer of postinfection ferret sera[a]				
				A/Wisconsin/67/05	A/Trieste/25c/07	A/Brisbane/10/07	A/Hong Kong/1985/09	A/Perth/16/09
Reference viruses								
A/Wisconsin/67/2005	8/31/2005	SpfCk3, E3 E4	−	**1,280**[b]	1,280	640	40	<40
			+	**1,280**	640	640	<40	<40
A/Trieste/25c/2007	January 2007	MDCKx MDCK4	−	40	**160**	160	80	80
			+	1,280	**1,280**	1,280	80	40
A/Brisbane/10/2007	2/6/2007	E2 E1	−	2,560	2,560	**2,560**	160	160
			+	1,280	1,280	**1,280**	<40	40
A/Hong Kong/1985/2009	4/1/2009	MDCKx2 SIAT2	−	40	40	80	**640**	320
			+	40	40	80	**1,280**	1280
A/Perth/16/2009	7/4/2009	E3 E1	−	<40	80	<40	640	**1,280**
			+	<40	<40	<40	640	**640**
Test viruses								
A/Paris/288/2009	11/10/2008	C2 SIAT1	−	40	40	80	40	<40
			+	1,280	2,560	1,280	40	<40
A/Perugia/5/2009	1/13/2009	MDCK1 SIAT1	−	80	80	160	40	40
			+	1,280	2,560	2,560	40	40
A/Johannesburg/251/2009	6/2/2009	MDCK1 SIAT1	−	80	80	80	80	80
			+	ND	ND	1,280	40	40
A/Singapore/33/2009	6/1/2009	E2 E1	−	40	40	80	80	160
			+	ND	ND	80	1,280	640
A/Hong Kong/2154/2009	5/18/2009	MDCK2 SIAT1	−	40	40	80	320	160
			+	ND	ND	160	5,120	640

[a] ND, not done.
[b] Homologous titers are shown in boldface type.
[c] Abbreviations: SpfCk, specific-pathogen-free chick kidney cells; E, embryonated egg; C, cell culture (unspecified); SIAT, MDCK-SIAT1 cells.

agglutination of guinea pig RBCs, due to HA binding, by egg-grown viruses and by MDCK cell-grown viruses in the presence of 20 nM oseltamivir carboxylate was well inhibited by high dilutions of anti-HA antiserum. However, agglutination by MDCK cell-grown viruses, which also agglutinated turkey RBCs, was poorly inhibited by anti-HA antiserum in the absence of oseltamivir. In contrast, the agglutination of turkey RBCs by the latter viruses was inhibited by anti-N2 antiserum but poorly by anti-HA antiserum (as for guinea pig RBCs), whereas agglutination by egg-grown viruses was not.

These results indicate that the agglutination of turkey RBCs by MDCK cell-grown viruses is due largely to NA binding, whereas both HA and NA contribute to the agglutination of guinea pig RBCs. The results thus provide an explanation for the differences in HI titers generally observed with HI tests using postinfection ferret antisera (Table 1). The results also indicate the relatively low levels of anti-NA antibody in the postinfection ferret antisera, previously apparent from results of neuraminidase inhibition (NI) tests.

Thus, the addition of 20 nM oseltamivir carboxylate to HI titrations with guinea pig RBCs to inhibit NA-dependent agglutination substantially increased HI titers of postinfection ferret antisera and improved the identification of and clarified the distinction between A/Brisbane/10/2007-like and A/Perth/16/2009-like viruses (Table 5).

Effects of mutations in HA and NA on hemagglutination. To demonstrate conclusively that NA was responsible for the oseltamivir-sensitive agglutination of turkey RBCs and to elucidate the effect of variations in residue 151, different combinations of HA and NA from selected viruses, in particular A/Hong Kong/4443/2005 and A/Trieste/25/2007, were inserted by reverse genetics into recombinant viruses together with the other six or seven genes of A/WSN/33.

The recombinant virus RG-Trieste/25(HAc), in which the HA of WSN was replaced by that of MDCK cell-grown A/Trieste/25c/2007, agglutinated guinea pig but not turkey RBCs, whereas RG-Trieste/25(HAe), which incorporated HA of the egg-passaged A/Trieste/25e/2007 (possessing the mutation L194P) agglutinated both species of cells (Table 3). Similarly, RG-Parma/1(HAe), containing HA (Nepal/921/06 clade) of A/Parma/1e/2007, which acquired the two amino acid substitutions H156R and K158N during egg passage, agglutinated both types of RBCs, whereas RG-Parma/1(HAc), with HA from the original MDCK cell isolate, agglutinated only guinea pig RBCs (Table 3). Thus, mutations (especially that encoding the L194P substitution) in HA acquired after passage in avian cells increased the capacity to agglutinate avian RBCs.

Of the two viruses incorporating NA of A/Trieste/25c/2007 with either D151 or G151 and HA of A/Trieste/25c/2007, the former virus, RG-Trieste/25(HAc/NA151D), agglutinated only guinea pig cells, as for the virus with HA alone; agglutination was not affected by the presence of 20 nM oseltamivir carboxylate, and virus was readily eluted from the RBCs within 3 h at room temperature. In contrast, the virus with the G151 mutation, RG-Trieste/25(HAc/NA151G), agglutinated turkey, chicken, human (and horse), as well as guinea pig RBCs, and agglutination was inhibited, or reduced in the case of guinea pig cells, by 20 nM oseltamivir carboxylate; virus was not eluted from the RBCs after 3, or even 7, days at room temperature. Similar results were obtained with RG viruses possessing equivalent HA and NA combinations of A/Hong Kong/4443/2005 (Table 3). Conversely, the RG-Finland/486(HAc/NAc) virus incorpo-

rating the HA and NA (with D151) of the earlier virus A/Finland/486/2004, like the wild-type parent virus, did not agglutinate chicken RBCs but agglutinated turkey and human as well as guinea pig RBCs, and agglutination was not affected by 20 nM oseltamivir carboxylate (Table 3). Since the only change in the NAs of A/Wisconsin/67/2005-like viruses relative to those of the earlier A/Wellington/1/2004-like viruses such as A/Finland/486/2004 was D93N, apart from the variation at position 151, it is apparent that the common changes in HA, S193F and D225N (Fig. 1A), were principally responsible for changes in hemagglutination.

Anti-HA antibody in hyperimmune sera or homologous postinfection ferret antisera was not effective in preventing the agglutination of guinea pig RBCs (or turkey RBCs) (data not shown) by the NA-151G viruses, in contrast to the NA-151D viruses, unless oseltamivir was included in the assay with guinea pig RBCs (Table 4). On the other hand, anti-NA antibody inhibited the agglutination of turkey RBCs (Table 4). The closer match between the NAs of A/Moscow/10/99 and A/Hong Kong/4443/2005 than between the NAs of A/Moscow/10/99 and A/Trieste/25/2007 can account for the higher HI titers observed for the anti-NA (A/Moscow/10/99) antibody against RG-HK/4443(HAc/NA151G) than against the RG-Trieste/25(HAc/NA151G) virus.

Partial sets of data obtained with different combinations of HA and NA, with G151 or D151, of other viruses of different genetic subgroups, e.g., A/Parma/1/2007 (Nepal/921/06 clade), were consistent with these results.

Thus, the D151G substitution is responsible for the acquisition of oseltamivir- and anti-NA antibody-sensitive hemagglutination and resistance to enzymatic elution. This finding indicates that the G151 NA attaches to receptors that are not cleaved by its enzyme activity and that attachment is inhibited by oseltamivir.

Effects of the D151G substitution on enzyme activity. Parameters of the neuraminidase activities of the RG-HK4443 viruses with D151 or G151 did not reveal any marked change as a result of the D151G substitution. With the substrate MUNANA, K_m values were 19.1 μM and 19.4 μM, respectively, and apparent V_{max} values were 3.1 and 4.3, respectively. Assays using fetuin as a substrate with the Amplex Red assay kit also showed no significant differences in enzyme activity. Furthermore, K_i values for competition by α2,3-sialyl-lactose (104 ± 16 μM and 220 ± 33 μM) and α2,6-sialyl-lactose (247 ± 35 μM and 312 ± 50 μM) were also similar for the D151 and G151 enzymes, respectively. The substitution D151G did, however, reduce susceptibility to oseltamivir and zanamivir by about 10-fold; K_i values were 0.09 nM (D) and 1.07 nM (G), respectively, for oseltamivir carboxylate and 0.20 nM (D) and 1.86 nM (G), respectively, for zanamivir.

Since it is apparent that the mutation does not alter the enzyme activity significantly, the specificities of the D151 and G151 enzymes were compared in reciprocal binding-elution experiments using the RG viruses with HA and NA of both A/Hong Kong/4443/2005 and A/Trieste/25/2007 viruses (Fig. 3). RBCs from which the D151 virus was eluted and that were refractory to agglutination by the further addition of D151 virus were shown to be agglutinated by the addition of the G151 virus; virus was not eluted by enzyme activity at 37°C for 3 h but was eluted by the addition of oseltamivir carboxylate

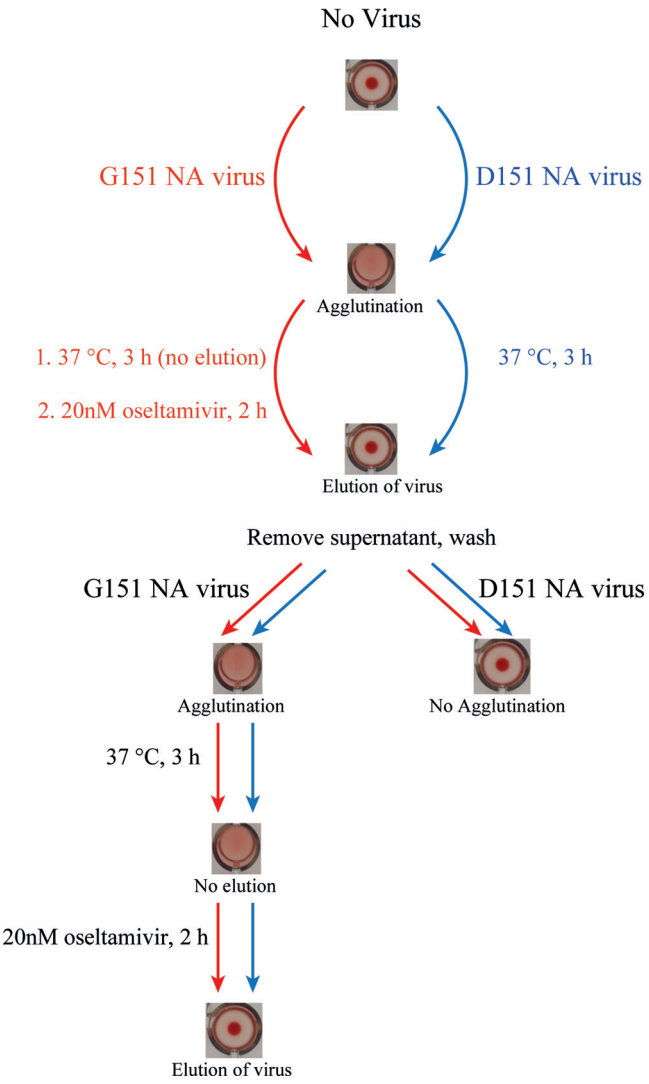

FIG. 3. Difference between receptors for HA and those for NA. Either RG-Trieste/25(HAc/NA) or RG-HK/4443(HA/NA) virus, with D151 or G151 in NA, were successively adsorbed to and eluted from guinea pig RBCs in reciprocal experiments. RBCs from the first adsorption-elution cycle with the D151 NA virus (blue pathway) or the G151 NA virus (red pathway) were washed three times with PBS before the addition of either virus. To elute viruses with G151 NA, agglutinated RBCs were treated with 20 nM oseltamivir carboxylate for 2 h at room temperature.

(20 nM for 2 h). In the reciprocal experiment, RBCs agglutinated by the G151 virus were incubated at 37°C for 3 h to allow time for the enzymatic removal of receptors for HA, and the virus was eluted by the addition of 20 nM oseltamivir carboxylate. The resulting cells were not agglutinated by the addition of the D151 virus but were reagglutinated by the addition of the G151 virus. These results demonstrate clearly that the neuraminidase activities of the viruses, with D or G at position 151 of NA, were similar in their capacities to remove receptors for HA and that the specificity of binding by the G151 NA is distinct from that of HA, with such receptor(s) being refractory to cleavage by either virus enzyme. The receptors for NA were removed by preincubating guinea pig (or turkey) RBCs with

the bacterial sialidases from *Clostridium perfringens*, *Vibrio cholerae*, and *Arthrobacter ureafaciens*, confirming that the attachment was to sialic acid moieties. The concentration dependence of the inhibition of agglutination by G151 and D151 viruses indicated that receptors for NA were removed less readily than those for HA.

DISCUSSION

The results of these studies demonstrate conclusively that the apparent inability of anti-HA antibody to inhibit hemagglutination by MDCK cell isolates of recent H3N2 viruses, indicated by the low titers in HI reactions with turkey RBCs, was the result of the attachment of virus via its NA due mainly to the replacement of Asp151 and not simply to an alteration of HA *per se*. Differences in HI reactions are, however, usually associated with differences in HA (antigenic or receptor binding), and it is apparent that a reduced avidity of receptor binding, exemplified by the loss of the ability of the HAs of these viruses to agglutinate turkey RBCs, underlies this phenomenon. Thus, the marked differences in inhibition of hemagglutination by MDCK cell- and egg-grown viruses by specific ferret antisera were due to differences in the selection of the viruses in different cells.

Passage in eggs selected, with difficulty, viruses with substitutions in HA1 (e.g., L194P was a common egg-selected mutation) that appear to increase the avidity of binding to and, consequently, the agglutination of avian RBCs (28, 30), which was readily inhibited by specific anti-HA antibody in postinfection ferret antisera. Comparisons of hemagglutination by egg- and MDCK cell-passaged viruses and recombinant viruses containing the HAs of either virus have indicated that such egg-selected changes complement the substitutions S193F and D225N in the receptor binding site of A/Wisconsin/67/2005-like viruses, which appear to have been responsible for reduced binding and the loss of the ability to agglutinate turkey, as well as chicken, RBCs compared to the earlier A/Wellington/1/2004-like viruses. Amino acid changes in residues 193 and 225 were previously shown to be capable of altering the specificity and/or affinity of receptor binding of H3 HAs (7, 23, 28, 30).

In marked contrast, a feature of many, if not most, MDCK cell isolates of H3N2 viruses circulating since 2005 was the relative inability of anti-HA antibody, either in postinfection ferret antisera or in polyclonal hyperimmune anti-HA antisera, to inhibit hemagglutination, particularly of turkey RBCs. On the contrary, inhibitors of NA activity, oseltamivir carboxylate and zanamivir, as well as anti-NA antibody inhibited the hemagglutination of turkey RBCs (and, to a lesser extent, guinea pig RBCs) by these viruses, whereas anti-HA antibody had little effect. Results were less clear-cut with guinea pig than with turkey RBCs since both HA and NA contributed to the agglutination of the former. The complete or partial replacement of D151 by G or N (or A in recently isolated viruses) was observed to correlate with the inhibition of hemagglutination by oseltamivir carboxylate and low HI titers in tests with postinfection ferret antisera. Thus, variation in nucleotide ambiguity in the codon for residue 151 of NA, resulting in various mixtures of viruses with D and G, N, or A at position 151, can readily account for the wide variation in results of HI tests, especially with turkey RBCs. It was apparent from comparisons of the amino acid sequences of HA and NA of virus isolates and the corresponding clinical specimens that passage in MDCK cells selected for these changes in NA and not for changes in HA, as recently reported by others (33, 36). Furthermore, the subsequent passage of MDCK cell isolates in embryonated eggs selected for receptor binding changes in HA and the loss of the mutation at position 151 in NA, emphasizing the differences in selective pressures in the two hosts and reflecting the better adaptation of HA to receptors on mammalian cells rather than to those on avian cells.

Incorporation of the two variant NAs with D or G at position 151 into recombinant WSN viruses demonstrated that oseltamivir-sensitive agglutination was due to G151. Furthermore, since the NAs with D151 or G151 were similar in their catalytic activities and in their capacities to remove HA receptors from RBCs, it is apparent that the D151G substitution does not simply prevent the cleavage of certain receptors but alters the specificity of NA, allowing it to bind additional receptors that are refractory to its catalytic activity. This property of NA therefore appears to complement a deficiency in HA binding.

The nature of the receptors has not been identified. They were readily removed by bacterial sialidases, and 10 μg/ml human glycophorin was effective in competing with NA binding, whereas higher concentrations (up to 2 mg/ml) of human α2-macroglobulin, bovine fetuin, and *E. coli* colominic acid, composed of poly-α2,8-linked sialic acid, had little effect. In some respects, the results of these limited studies reflect those reported previously by Gulati et al. (11), who observed that glycophorin was a more potent inhibitor of hemagglutination by an A/Fujian/411/2002-like virus and concluded that "specific forms of sialic acid are used as receptors by recent H3N2 viruses" but did not identify NA as being responsible for binding.

The location of residue 151 in the 150 loop at the edge of the active site of NA (Fig. 4) (6, 35, 40) and the sensitivity of binding to oseltamivir indicate that binding involves the catalytic site and not a second sialic acid binding site analogous to that identified on NAs of avian influenza viruses (19, 42). Furthermore, since the concentration dependence of the inhibition of attachment by oseltamivir carboxylate was similar to that of the inhibition of enzyme activity, it is apparent that NA binds with a similar affinity. The location of the second sialic acid binding site adjacent to the catalytic site was identified by the X-ray crystallographic structure of sialic acid bound to the second site of N9 NA, together with the influence of mutations that indicated the minimum requirements for binding to be a combination of the triple serines 367, 370, and 372 within the loop at residues 367 to 373, asparagine 400, and tryptophan 403 (17, 32, 38, 39). The L370S and S372L substitutions were features of the NAs of viruses that emerged during 2006 (Fig. 1B). Although some "intermediate" viruses, such as those of the Nepal/921/06 clade, possessed the triple-serine motif, they lacked residues N400 and W403, which were shown to be essential for the hemadsorption activity of N2 NA (38). Hemadsorption via this site was also observed not to be sensitive to inhibitors of catalytic activity (17, 19). Furthermore, the proposed role of the hemadsorption site in increasing the catalytic efficiency of the enzyme (38) and not in virus attachment

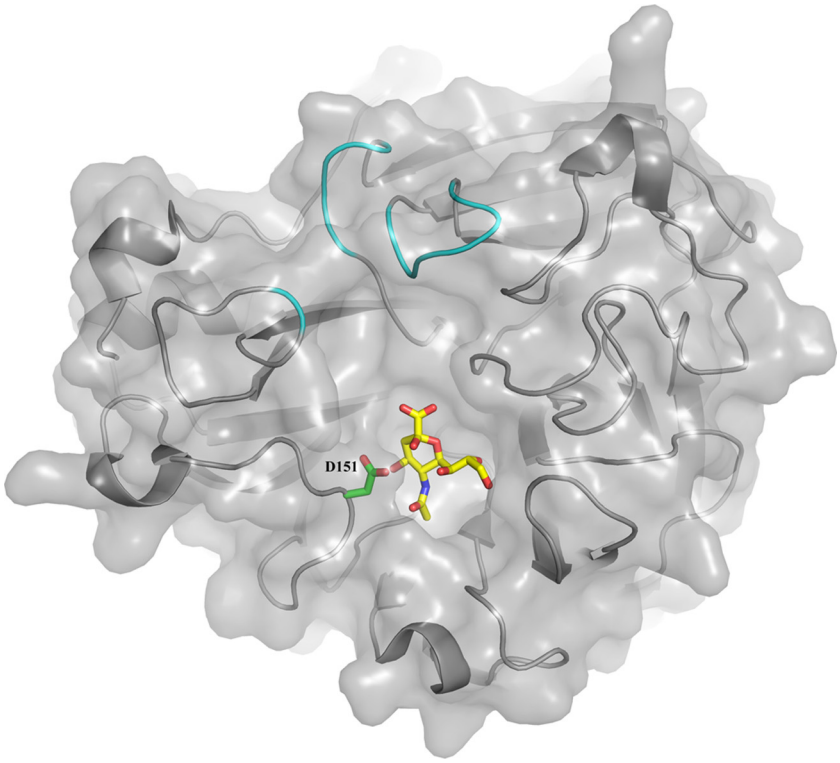

FIG. 4. Location of residue 151 (green) in relation to the catalytic site of NA. Sialic acid (yellow) is located in the X-ray crystal structure of one subunit of the NA tetramer of A/Tokyo/3/67 (40). Residues 367 to 372, 400 to 403, and 432, forming the hemadsorption site of avian influenza virus NAs, are shown in blue.

is inconsistent with the apparent role of NA-dependent binding in virus attachment reported here.

A variety of structural and mechanistic studies have indicated that the principal role of aspartic acid 151 is to stabilize the transition-state intermediate in the cleavage reaction (4, 10, 15). However, the similarities in the activities of the G151 and D151 enzymes indicate that aspartic acid is not essential for catalysis. Since a replacement by asparagine as well as glycine or alanine allows the enzyme to bind a sialyl receptor that it cannot cleave, it appears that the aspartic acid does have an important role in restricting the binding specificity of NA to match both its cleavage activity and the complementary binding specificities of HA. In this respect, extended passage in MDCK-SIAT1 cells of the recombinant viruses with G151 led to a partial reversion and mixed populations of G151 and D151 viruses by passage 5, indicating the complementary nature of the two versions of NA. After a single passage in eggs, the viruses possessed mainly D151. These observations emphasize the delicate balance between receptor binding and receptor removal, in terms of both the specificities and activities of the virus proteins, for optimal replication.

Although the identity of the sialyl receptor(s) is unknown, the interaction of Asp151 with the hydroxyl on C4 and the glycosidic linkage (10, 40) suggests the possibility that substitution may allow the binding of four substituted sialic acids or sialic acids in a different linkage to the penultimate sugar. Preliminary observations that changes in other residues in the 150 loop, in particular T148I, also appear to be associated with NA-mediated agglutination suggest a more general role for the conformation of the 150 loop, shown to be flexible in group 1 NAs (35), in determining the specificity of sialoside interaction.

The biological *in vivo* significance is not clear. It is apparent from the limited pyrosequencing studies reported here and reports from others (33) that viruses present in clinical samples contain, at most, relatively low levels of the G, N, or A substitutions at position 151 of NA. However, the relative ease of selection of these mutations during passage in MDCK cells, in preference to receptor binding mutations in HA, does indicate how they might complement the low avidity of HA. Although the selection of these mutations during passage in MDCK cells was more common following the emergence of the A/Wisconsin/67/2005-like viruses with the changes at positions 193 and 225 in HA and no distinctive change in NA, this phenomenon was not peculiar to these viruses. NA D151G substitutions or mixtures of D and G or N at position 151 of NAs among H3N2 viruses were reported previously (27) and were identified by examination of sequences of H1N2 viruses conducted during the present studies as well as those recently reported (33, 36). However, the possibility that such a phenomenon might participate in the immune evasion of anti-HA antibody is not supported by the demonstration that although anti-HA antibody failed to prevent hemagglutination by viruses possessing mixtures of G151 and D151 (Table 1), it was effective in neutralizing virus infectivity (Table 2) by inhibiting the essential functions of HA in virus entry.

From a practical perspective, we have identified a phenomenon that has compromised the usefulness of the simple HI

test as a surrogate for virus neutralization in comparisons of antigenic relatedness and shown how this can be circumvented by the inclusion of an inhibitor of the confounding NA-hemagglutinating activity. On the other hand, changes in HA binding, which caused a loss of agglutination of turkey RBCs, did not compromise the usefulness of the HI assay using guinea pig RBCs for comparisons of the antigenic characteristics of recently isolated H3N2 viruses. Although substitutions in residue 151 have relatively little effect on the NA inhibitor sensitivity of the isolates (27, 33), other mutations selected during *in vitro* passage, such as the Q136K substitution, which substantially reduces zanamivir susceptibility (14, 33), may give misleading conclusions as to the drug susceptibility of the natural virus. In relation to the production of cell culture-based vaccines, it is reassuring that mutations are selected in NA rather than HA, with less potential impact on the antigenic characteristics of the vaccine. The results of these studies do, however, emphasize once again the importance of monitoring cell adaptive changes and understanding the features of viruses that are important for optimal growth in different cell substrates.

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